J Chem Ecol (2009) 35:698–705
DOI 10.1007/s10886-009-9650-4

Comparison of Age-dependent Quantitative Changes in the Male Labial Gland Secretion of *Bombus Terrestris* and *Bombus Lucorum*

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Received: 12 March 2009 / Revised: 27 May 2009 / Accepted: 2 June 2009 / Published online: 20 June 2009

Abstract Age-related changes of antennal-active components of male labial gland extracts were studied in two closely related bumblebee species, *Bombus terrestris* and *B. lucorum*. In *B. terrestris,* compounds eliciting electroantennogram (EAG) responses of virgin queens were ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecan-1-ol, octadeca-9,12,15-trien-1-ol, and geranylcitronellol. Compounds that elicited EAG responses from queens of *B. lucorum* were ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecan-1-ol, hexadec-7-enal, octadeca-9,12-dien-1-ol, octadeca-9,12,15-trien-1-ol, and octadecan-1-ol. Quantities of these compounds in the labial glands changed significantly over the lifetime of the respective males of the two species. In both species, concentrations of the respective compounds reached their maximum within seven days after eclosion. Subsequently, a rapid decrease in the amount of EAG-active compounds occurred in *B. terrestris*, whereas in *B. lucorum* the amount of active compounds stayed approximately constant or decreased at a slow rate. Microscopy showed that in *B. terrestris* secretory cells of the labial glands undergo apoptosis from the fifth to the tenth day of life, whilst in *B. lucorum* labial gland cells remain unchanged throughout the life of the males.

Keywords Bumblebee · *Bombus terrestris* · *Bombus lucorum* · Labial gland · Male marking pheromone · Sex pheromone · GC-EAD

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Introduction

The most common European bumblebee species, *Bombus terrestris* (Linnaeus 1758) and *Bombus lucorum* (Linnaeus 1761), both belong to the subgenus *Bombus sensu stricto*. As observed for other bumblebee species during premating behavior, *B. terrestris* and *B. lucorum* males scent-mark prominent objects on their flight routes with a species-specific sex pheromone ('patrolling behavior'; Calam 1969; Schremmer 1972; Svensson 1980; Lloyd 1981; Morse 1982; Villalobos and Shelly 1987) that attracts conspecific virgin queens to the marked spots for mating (Kullenberg et al. 1970; Bergström et al. 1981; Bergman 1997).

The male-produced pheromones of these bumblebees are produced in the cephalic part of the labial gland (Kullenberg et al. 1973; Bergman and Bergström 1997). Volatile components of the gland secretion are deposited on prominent objects on the flight routes or on perches (Bergman and Bergström 1997; Kindl et al. 1999). The secretion is a complex mixture, comprised of many compounds, usually with one or two major components (Valterová and Urbanová 1997; Terzo et al. 2003). In closely related bumblebee species, the secretions may be similar but usually differ in dominant components and/or compound proportions. Although the main components of the secretion are presumed to comprise the sex pheromone, behavioral roles of specific components in female attraction have yet to be demonstrated. Thus, the actual male-produced pheromone components in secretions of bumblebee species are unknown.

The composition of bumblebee labial gland secretions is used as a tool for taxonomic identification and species and subspecies discrimination (Paterson 1993; Terzo et al. 2005; Rasmont et al. 2005; Bertsch et al. 2005; Coppée et al. 2008). However, there is great inter-individual variability in the composition of secretion components within a single species (Svensson and Bergström 1977; Ågren et al. 1979; Šobotník et al. 2008), which can make differentiation difficult, especially among related species. Recently, changes of cephalic labial gland ultrastructure in relation to age of males were reported for *B. terrestris* (Šobotník et al. 2008). It was found that the secretory activity of the gland cells is high in newly emerged males, but drops as males age. Five days after eclosion of the adult male bumblebee, the biosynthetic activity within the gland stops, and the secretory cells degenerate (Šobotník et al. 2008). Maximal gland content occurred in 2–7 day-old males, and decreased in older males. This age-related change in gland content was paralleled by the ability of gland extracts from different age males to elicit electroantennogram (EAG) responses from queen antennae. Although the study by Šobotník et al. (2008) demonstrated changes in the volume of gland content with age, there was no detailed chemical analysis of changes in the quantities of components of the secretion. They did, however, perform coupled gas chromatogram-electroantennogram detection (GC-EAD) experiments, using male labial gland secretions and queen antennae, and found at least six antennal-active compounds, ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadecatrienol, and geranylcitronellol, that could potentially play a role in male sex pheromone signaling (Šobotník et al. 2008; Valterová et al. 2007).

Qualitative analysis of the labial gland extract from *B. lucorum* has previously been performed (Bergström et al. 1973; Urbanová et al. 2001). However, no attention has been paid to age of males. There are a few reports on seasonal variation in the secretion composition of other bumblebee species (Kullenberg et al. 1970; Svensson and Bergström 1977; Ågren et al. 1979), but thus far the phenomenon of age-related changes in composition has not been studied systematically.

In the present study, we report age-dependent quantitative changes of major EAG-active components of the labial gland of two related bumblebee species, *B. lucorum* and *B. terrestris*. This allowed us to determine whether the relatively time-limited labial gland secretion activity observed in *B. terrestris* also occurs in another species in the subgenus *Bombus sensu stricto.*

Methods and Materials

Insects Colonies of *B. lucorum* and *B. terrestris terrestris* (L.) were established by the two-queens cascade method (Ptáček et al. 2000). All mother queens were taken from their natural habitats during the nest-searching period in order to minimize the possible negative influence of artificial conditions on the progeny. Bumblebee colonies were kept in plastic boxes of 0.6–1 L volume and fed with honeybee pollen pellets and concentrated sugar solution (sucrose:fructose 1:1). When colonies started to produce males, male cocoons were removed from the parental hives and left to mature separately under the care of several workers supplied with food (Ptáček 1999). Freshly emerged males were removed and kept according to age. Animals of the following age were studied. *Bombus terrestris*: shortly after eclosion, 1, 2, 3, 4, 5, 7, 10, 12, 17, 20, 24, and 33 days after eclosion (five individuals of each age); *B. lucorum*: shortly after eclosion, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, and 30 days after eclosion (5–6 individuals of each age). Males were killed by freezing (−18°C) and were kept frozen prior to dissection. Labial glands were dissected and extracted with hexane (100 µl per gland) containing 1-bromodecane as internal standard (1.79 mg/ml for *B. terrestris* and 2.13 mg/ml for *B. lucorum*). Glands, in solvent, were shaken for 30 min after which the extracts were transferred to clean vials and kept at −18°C prior to analysis.

Identification of Compounds Extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) using a splitless injector (200°C), mass detector (200°C, Fisons MD 800) and autosampler AI3000 (Thermo). A DB-5 ms column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies) and helium gas (constant flow of 1 ml min^{-1}) were used for separations. The temperature programs differed for samples of the different species: for *B. terrestris*, 70°C (2 min. delay) to 320°C at 10°C min^{-1}; for *B. lucorum*, 70°C (2 min. delay) to 140°C at 40°C

min^{-1}, to 240°C at 2°C min^{-1}, and then to 320°C at 4°C min^{-1}. Compounds were identified based on their mass spectra and on co-chromatography with synthetic or commercially available standards.

Gas chromatography-electroantennogram detection GC-EAD experiments were performed with a 5890A Hewlett-Packard gas chromatograph (GC) equipped with a DB-5 column (30 m×0.25 mm, film thickness 0.25 µm, J & W Scientific). The column was split by a Graphpack 3D/2 four-arm splitter. The splitter led the eluate to flame ionization (FID) and EAD detectors. N_2 make-up gas at 20 ml min^{-1} was introduced via one arm of the splitter. Labial gland extracts (1–5 µl) were injected in the splitless mode. The GC was temperature programmed from 50°C (2 min. delay) to 270°C at 30°C min^{-1}. The temperature of the injector and FID were set to 230 and 260°C, respectively. The EAD detector consisted of a queen antenna connected via two glass Ag/AgCl electrodes to a universal AC/DC 10XProbe (Syntech, Hilversum, The Netherlands). The EAD and FID signals were fed to a computer via the serial IDAC interface box (Syntech) and analyzed by using GC-EAD software (Syntech). The antenna was exposed to compounds that eluted from the GC via an Effluent Conditioner Tube (Syntech) heated to 180°C. Virgin queens (N=4) used for GC-EAD recording were kept at low temperature (5°C) and high humidity until use. Isolated antennae, with the distal tips excised, were used for recordings. Each antenna was used only once.

Chemicals The following standards were used for the quantification of EAG-active components of the labial gland secretions: (*E*)-farnesol (Firmenich, Geneva, Switzerland), geranylgeraniol (ICN, Irvine, CA, USA), ethyl tetradec-9-enoate (Nu-Check-Prep, Elysian, MN, USA). (*Z,Z,Z*)-Octadeca-9,12,15-trien-1-ol, ethyl dodecanoate, hexadecan-1-ol, octadecan-1-ol, (*Z,Z*)-octadeca-9,12-dien-1-ol, and ethyl hexadec-9-enoate were purchased from Sigma (St Louis, MO, USA). (*Z*)-Hexadec-9-enal was prepared earlier in our laboratory.

Quantitative Analyses Only EAD-active compounds were quantified. For *B. terrestris*, 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, geranylgeraniol, geranylcitronellol, (*Z,Z,Z*)-octadeca-9,12,15-trien-1-ol, and ethyl dodecanoate were quantified; *B. lucorum:* ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadecan-9,12-dienol, octadecan-9,12,15-trienol, and octadecanol were quantified. Quantification was carried out in Total Ion Current mode of the mass detector and based on peak areas. Because the different compounds were present in samples at widely differing concentrations, we had to avoid overloading the mass detector with the most abundant component. Separation of ethyl tetradec-7-enoate and ethyl tetradec-9-enoate under the conditions used was poor. Therefore, these two isomers were quantified together according to the external calibration for ethyl tetradec-9-enoate. Previous studies (Urbanová et al. 2001) showed that the concentration ratio of these isomers in the labial gland extracts was: $\mathrm{area}_{\mathrm{ethyltetradec-9-enoate}} / \mathrm{area}_{\mathrm{ethyltetradec-7-enoate}} = 187.5$. We assumed that the difference in responses of these isomers in the mass detector was less than 5%, and the concentration ratio remained constant over a bumblebee's life.

The calibration curve for (*Z*)-hexadec-7-enal was constructed by using (*Z*)-hexadec-9-enal, as the correct isomer was not available. 2,3-Dihydrofarnesol and 2,3-dihydrofarnesal quantification was calibrated by using farnesol, and geranylcitronellol was calbrated by using geranylgeraniol, due to unavailability of standards. Considering the close similarities in structures between calibrants and compounds, we assumed the quantification error to be low. Octadeca-9,12-dienol and octadeca-9,12,15-trienol could not be separated, but the same approach and assumption as used for ethyl tetradec-7-enoate and ethyl tetradec-9-enoate were used for the samples and quantitative calibration.

The same internal standard (IS; 1-bromodecane, ~2 mg/ml) was used for both calibration and sample sets. Calibration curves were constructed from peak area (ratios of standard/IS). Second degree polynomial equations were used to fit the obtained data. Unknown sample concentrations were calculated by using the calibration curve equations and expressed in µg per gland for each component. Mean values and standard errors were calculated for each male age group.

Microscopy Fixed cephalic parts of labial glands of *B. lucorum* originated from males of ages, pharate imago (darkly colored males enclosed in pupal cuticle shortly before eclosion), <1 d, 1, 2, 3, 4, 5, 6, 9, and 13 days. Two to four samples of each age were studied using optical microscopy and, as only slight differences were observed over the adult life of a male, only a single sample of each age was studied by transmission electron microscopy. Dissection took place in a droplet of fixative (2% glutaraldehyde and 2.5% formaldehyde in 0.1 M phosphate buffer), in which the tissue stayed for 1 day at ambient temperature. After post-fixation in 1.5% OsO_4 in 0.1 M phosphate buffer, the samples were dehydrated by passing through a series of ethanol-water mixtures (50, 75, and finally 100%). Tissues were then embedded into standard Spurr resin. Semithin sections (1 µm) were stained with Azure II and observed under Amplival (Zeiss) optical microscope (equipped with Canon EOS 300D camera). Ultrathin sections were studied with a Jeol 1011 transmission electron microscope.

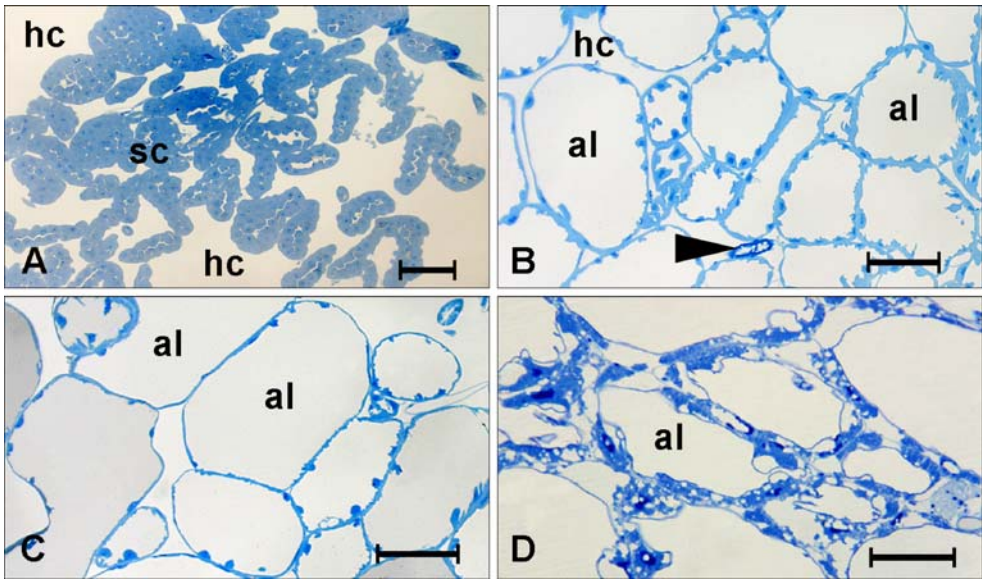

Fig. 1 Development of acini in the cephalic part of the labial gland of *Bombus lucorum* (A–C) and *B. terrestris* (D) (optical microscopy); **a** development of acini in a 1-day-old adult male of *B. lucorum*; **b** development of acini in a 3-day-old male of *B. lucorum*. The arrowhead marks an excretory duct of the labial gland; **c** development of acini in a 13-day-old male of *B. lucorum*; **d** development of acini in a 13-day-old male of *B. terrestris* (all cells are already dead, but the acini are still full of secretion). Bar represents 100 µm in all figures. Abbreviations: al, acinar lumen; hc, haemocoel; sc, secretory cells

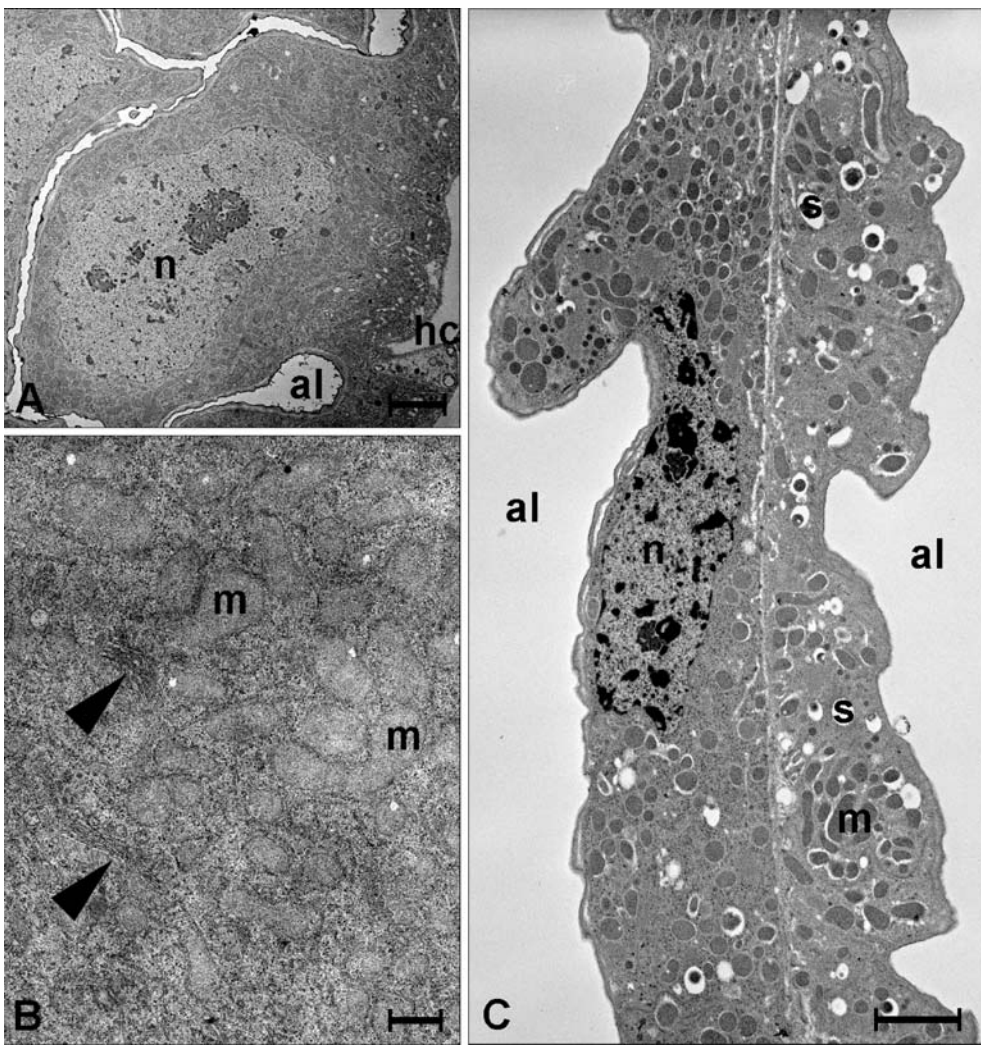

Fig. 2 Transmission electron micrographs of acinar secretory cells in *Bombus lucorum*; **a** whole secretory cell in pharate male imago. Note the active transport at the cell base occurring as pinocytotic vesicles. Bar represents 2 µm; **b** detail of cytoplasm in <1d adult male. Arrowheads mark smooth endoplasmic reticulum producing a secretion. Bar represents 500 nm; **c** walls of two neighbouring acini in a 13-day old male. Bar represents 2 µm. Abbreviations: al, acinar lumen; hc, hemocoel; m, mitochondria; n, nucleus; s, secretion within the cells

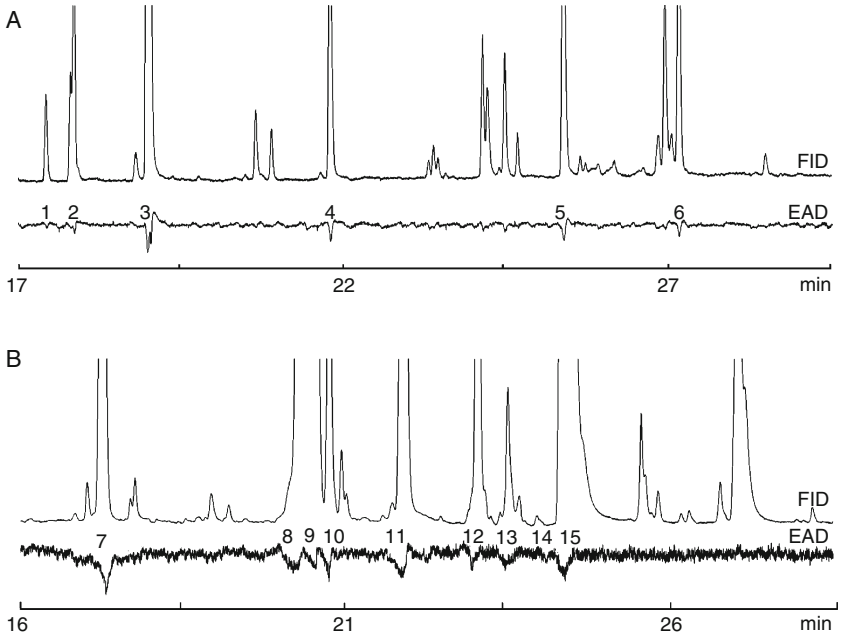

Fig. 3 Coupled gas chromatograph-electroantennogram detection recording of the labial gland extract of 7-day-old bumblebee males. **a** *Bombus terrestris*; active compounds: **1** = ethyl dodecanoate; **2** = 2,3-dihydrofarnesal; **3** = 2,3-dihydrofarnesol; **4** = hexadecan-1-ol; **5** = octadeca-9,12,15-trien-1-ol; **6** = geranylcitronellol. **b** *B. lucorum*; active compounds: **7** = ethyl dodecanoate, **8** = ethyl tetradec-7-enoate, **9** = ethyl tetradec-9-enoate, **10** = hexadec-7-enal; **11** = ethyl hexadec-9-enoate; **12** = hexadecan-1-ol; **13** = octadeca-9,12-dien-1-ol; **14** = octadeca-9,12,15-trien-1-ol; **15** = octadecan-1-ol. FID (upper trace) = flame ionixation detection, EAD (lower trace) = electroantennogram detection

Results

Gland Microscopy Microscopy of the cephalic labial gland showed differences between the two species. Unlike *B. terrestris*, the labial gland of *B. lucorum* remained functional throughout the life of the adult male. Additionally, in *B. lucorum*, the production of lipid secretion by the smooth endoplasmic reticulum (SER) started earlier (at time of emergence) (c.f., in *B. terrestris* production started on the 2nd day after emergence). The lipids were continuously secreted at the cell apices and, as their volume increased, were accompanied by swelling of the lumen and a decrease in cell layer thickness (Fig. 1a–c; optical microscopy).

The active transport of precursors from the hemolymph was observed in young males of both species, with the transport stopping during the third day after eclosion in *B. terrestris* (Šobotník et al. 2008), but continuing in males of *B. lucorum* up to the 13th day after eclosion. This

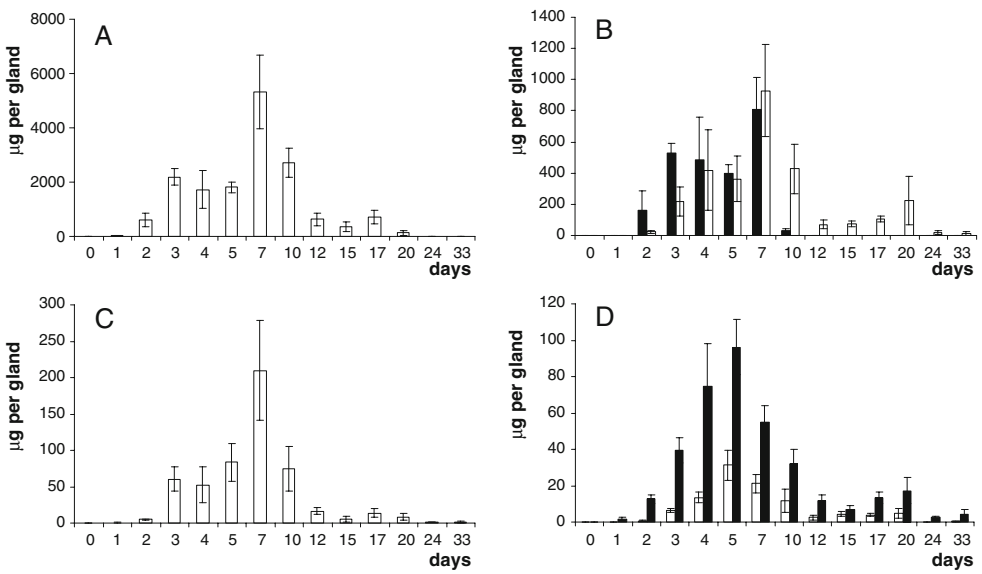

Fig. 4 Concentration changes, with regard to age of adult male, of electroantennogram-active compounds in the cephalic labial gland of *Bombus terrestris* males (mean ± standard error). **a** 2,3-dihydrofarnesol; **b** geranylcitronellol (white bars) and ethyl dodecanoate (black bars); **c** hexadecan-1-ol; **d** 2,3-dihydrofarnesal (white bars) and octadeca-9,12,15-trien-1-ol (black bars)

difference is associated with the different fates of the secretory cells in both species. The cells died after several days of secretory activity in *B. terrestris* (between the 5th and 10th day, Fig. 1d), whereas they conitnued to produce secretion throughout the life of adult *B. lucorum* males. However, even in older *B. lucorum* males, flattening of cells, lower volume of SER, and fewer droplets of secretion in the cell cytoplasm were observed (Fig. 2a,b; electron microscopy) indicating a decreasing rate of secretion production.

GC-MS, GC-EAD, and quantitative analyses In the labial gland of *B. terrestris,* the EAD-active compounds were identified as ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadeca-9,12,15-trienol, and geranylcitronellol (Fig. 3a). In *B. lucorum*, ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadeca-9,12-dienol, octadeca-9,12,15-trienol, and octadecanol elicited antennal responses in conspecific queens (Fig. 3b).

The EAD-active compounds of both species were quantified. In *B. terrestris*, the most abundant EAD-active component of the extract was 2,3-dihydrofarnesol (Fig. 4a). Males produced small quantities (ca. 20 µg/gland) of this compound on the day of eclosion. The quantity of this compound increased up to 2 mg/gland over the next two days, and remained roughly constant at this level for three more days. On the 6th and 7th days following eclosion, the concentration increased almost three times to ca. 6 mg/gland. Subsequent to this, the amount of 2,3-dihydrofarnesol decreased dramatically, such that at 10 days after eclosion, it fell to ca. 0.5 mg/gland. By day 20, 2,3-dihydrofarnesol was present in the gland at a very low amount (2 µg/gland). A similar pattern was observed for geranylcitronellol (1 µg–1 mg/gland, Fig. 4b) and hexadecanol (0.5 µg–200 µg/gland, Fig. 4c). A different age-dependent pattern was observed for ethyl dodecanoate, which appeared in the secretion later than the terpenes (at 2 days, 0.5 µg/gland), increased to its maximum concentration (800 µg/gland) on day 7, and then dropped to 0.7 µg/gland by day 20 (Fig. 4b). The remaining EAD-active compounds, octadecatrienol and 2,3-dihydrofarnesal, reached a maximum concentration around day five (100 µg/gland and 30 µg/gland, respectively, Fig. 4d).

The concentration profiles of the most abundant EAD-active compounds (i.e., ethyl tetradec-7-enoate and ethyl tetradec-9-enoate) present in the gland of *B. lucorum*, (Fig. 5a), differed from those in *B. terrestris.* In *B. lucorum*, no significant maxima were observed in the profile of the most abudnant EAD-active compounds, with the quantities remaining relatively constant over the adult life of the males. Ethyl tetradec-7-enoate and ethyl tetradec-9-enoate were both present (ca 0.9 mg/gland) in the gland on the day after eclosion, with maximum quantities (total of 1.5 mg/gland) occurring between days 7–20. The quantities of these two compounds declined significantly by day 25. Other EAD-active compounds, ethyl dodecanoate and hexadecanol (Fig. 5b), were present at roughly one tenth the amount (113 and 91 µg/gland, respectively) of the main component, but their concentration profiles exhibited a similar pattern with no significant maximum or changes between days 4–10. However, by day 15, the amount of hexadecanol increased, especially in comparison to ethyl dodecanoate, and reached a maximum in 25-day old males (185 µg/gland). Patterns in concentration changes similar to that of hexadecanol were observed for ethyl hexadec-9-enoate and hexadec-7-enal (Fig. 5c). The alcohols octadeca-9,12-dienol, octadeca-9,12,15-trienol (Fig. 5d),

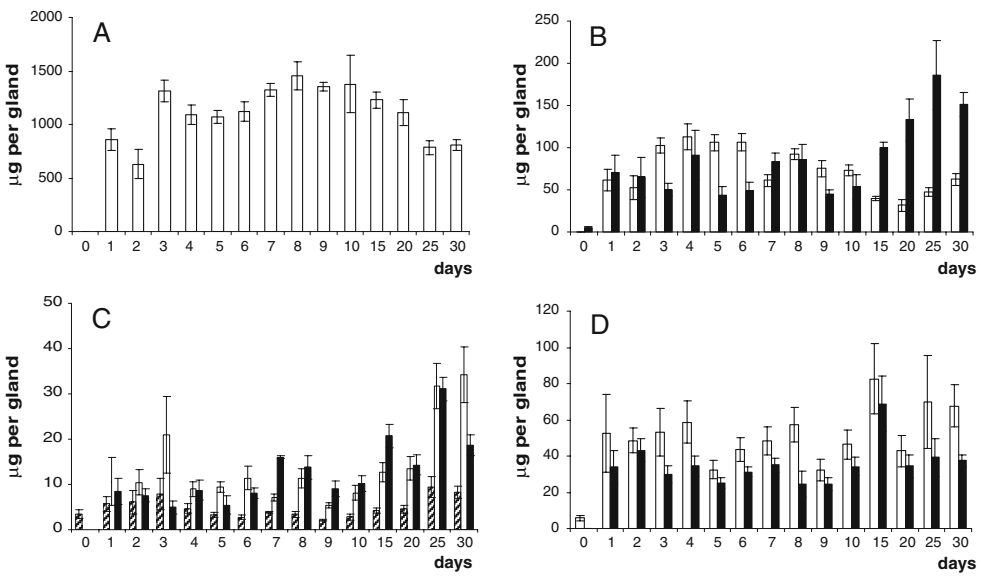

Fig. 5 Concentration changes, with regard to age of adult male, of electroantennogram-active compounds in the cephalic labial gland of *Bombus lucorum* males (mean ± standard error). **a** total ethyl tetradec-7-enoate and ethyl tetradec-9-enoate; **b** ethyl dodecanoate (white bars) and hexadecan-1-ol (black bars); **c** octadecan-1-ol (hatched bars), ethyl hexadec-9-enoate (white bars), and hexadec-7-enal (black bars); **d** octadeca-9,12-dien-1-ol (white bars) and octadeca-9,12,15-trien-1-ol (black bars)

and octadecanol (Fig. 5c) remained at a similar concentration (30–70 μg/gland) throughout the life of the male.

Discussion

The patterns in the quantitative profiles of the various EAD-active compounds corresponded well with the physiological and morphological changes observed in the labial glands for both species. While in *B. lucorum*, the secretory activity of the acinar cells continued over the lifetime of adult males, in *B. terrestris*, production stopped in males older than 10 days. The reasons behind the profound differences in labial gland morphlogy and chemical production between the closely related species are not clear and cannot be explained by lifetime of males, since laboratory observations show no difference in longevity (~50 days) of both species.

We speculate that the age-dependent changes in gland content may play a role in mate selection. Maximal labial gland content (and activity of secretory cells) was found in 3–5 day-old males of *B. terrestris*, which corresponds well with the time when males usually leave their natal nests (fifth day after eclosion) and start to mark and patrol. Additionally, maximal content in the gland matches maximal sperm content (6th day post-emergence; Tasei et al. 1998). In the laboratory, *B. terrestris* males mate between day 6–27 with the probability of successful mating dropping dramatically after day 11 (Tasei et al. 1998). Information about optimal reproductive behavior and physiology in *B. lucorum* is not available, but it would be interesting to know whether it matches the greater duration of labial gland content of EAD-active compounds found in this study.

It has been hypothesized that virgin queen bumblebees searching for a mate are likely to be more attracted to locations where higher amounts of secretion have been deposited by patrolling males (Ågren et al. 1979). Thus, given our results, it is possible that younger males of *B. terrestris* may have an advantage, over older males, in attracting queens, because their scent marks are likely to contain higher amounts of labial gland secretions than those of older males. Such an advantage would not be expectecd in *B. lucorum*, since young and old males have similar quantities of labial gland secretions.

Earlier reports on the variation in composition of the labial gland secretions of bumblebees are scarce. Kullenberg et al. (1970) described considerable seasonal variation in the diterpene content in the glands of *B. hortorum* and *B. hypnorum*. Svensson and Bergström (1977) found that diterpenic components in the labial gland secretion of *B. pratorum* males appear later in the season. Ågren et al. (1979) studied changes in labial gland secretion of various bumblebees species. No labial gland secretion compounds were observed in pupae of *B. hypnorum*. The compounds appeared from day 1 after emergence, and their amounts increased rapidly over the next 4–7 days. For *B. lapidarius*, measurements of gland content were not made until 7 days after eclosion. Secretion content had dropped 14 days after eclosion and further declined to undetectable by 19 –29 days. A similar declining trend was observed for *B. hortorum* males (Ågren et al. 1979). Although this study utilized an analytical technique (TLC) that did not allow precise quantifications of compounds to be made, the results are consistent with our observations for *B. terrestris*.

Although attractiveness of the male labial gland secretion for young queens has been demonstrated by Bergman (1997), roles of individual components in the blend have not been studied. Our study on the quantitative changes of EAG-active chemicals has identified a number of potential pheromone components, which should be tested in bioassays.

Finally, our results show that the production and composition of the labial gland secretion of male bumblebees vary considerably with age. Thus, chemotaxonomic studies that use labial gland secretions to distinguish between closely related species should take this variation into account, perhaps by analyzing solely mature and sexually active males. Such chemotaxonomic studies are best combined with other techniques such as morphology and genetic analysis, for differentiating clsoely related species (Bertsch et al. 2005).

Acknowledgements This work was financially supported by the Ministry of Education of the Czech Republic (project No. 2B06007), by the Czech Science Foundation (project No. 203/09/1446), and by the Academy of Sciences of the Czech Republic (research project No. Z40550506). All are gratefully acknowledged. A.C. and F.V. thank Fonds dela recherche fondamentale et collective (project No. 2.4.564.06.F) for support. Authors are grateful to J. Pflegerová (Institute of Entomology, Academy of Sciences of the Czech Republic, České Budějovice) and to the staff of the Laboratory of Electron Microscopy (Faculty of Sciences, Charles University, Prague) for their kind help. The authors would also like to thank A. Nekolová and M. Kojecká for their careful technical assistance, J. Cvačka and J. Zichová for valuable advice on analytical matters, and M. Foley for language consulting and comments on the manuscript.

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